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Molecular and biological characterization of a *Cypovirus* from the mosquito *Culex restuans*

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Abstract

A cypovirus from the mosquito *Culex restuans* (named CrCPV) was isolated and its biology, morphology, and molecular characteristics were investigated. CrCPV is characterized by small (0.1–1.0 μm), irregularly shaped inclusion bodies that are multiply embedded. Laboratory studies demonstrated that divalent cations influenced transmission of CrCPV to *Culex quinquefasciatus* larvae; magnesium enhanced CrCPV transmission by ∼30% while calcium inhibited transmission. CrCPV is the second cypovirus from a mosquito that has been confirmed by using molecular analysis. CrCPV has a genome composed of 10 dsRNA segments with an electropherotype similar to the recently discovered UsCPV-17 from the mosquito *Uranotaenia sapphirina*, but distinct from the lepidopteran cypoviruses BmCPV-1 (*Bombyx mori*) and TnCPV-15 (*Trichoplusia ni*). Nucleotide and deduced amino acid sequence analysis of CrCPV segment 10 (polyhedrin) suggests that CrCPV is closely related (83% nucleotide sequence identity and 87% amino acid sequence identity) to the newly characterized UsCPV-17 but is unrelated to the 16 remaining CPV species from lepidopteran hosts. A comparison of the terminal segment regions of CrCPV and UsCPV-17, an additional method for differentiating various *Cypovirus* species, revealed a high level of conservation. Therefore, we propose that CrCPV is a member of the *Cypovirus*-17 group and designate this species as CrCPV-17.

Keywords: *Culex restuans Cypovirus*; Transmission; Divalent cations; Mosquito; Morphology; Genomic; Electropherotype

1. Introduction

Occluded RNA viruses of insects (formerly cytoplasmic polyhedrosis viruses) have been classified in the family *Reoviridae* within the genus *Cypovirus* (CPVs). CPV virions are embedded (occluded) by a polyhedrin protein to form the resulting inclusion body (Xia et al., 1991). Zhang et al. (1999) reported that CPVs have a single-shelled capsid instead of a multiple-shelled organization as found in other *Reoviridae* members. These icosahedral, cubic, or sometimes irregular particles infect midgut cells of a wide range of insects (Payne and Rivers, 1976). After ingestion of the inclusion bodies by the insect, the alkaline pH and proteinases of the intestinal track break down the polyhedrin and release the virions, which enter and replicate in the cytoplasm of midgut epithelial cells (Mertens et al., 1999, 2004b; Zhang et al., 1999). Most CPV infections produce chronic disease with low mortality although some are pathogenic (Mertens et al., 1999, 2004b).

CPV genomes usually consist of 10 double-stranded RNA (dsRNA) segments (Seg-1 to Seg-10) (Fujii-Kawata et al., 1970). An eleventh segment has been shown in cypoviruses from TnCPV-15 (*Trichoplusia ni*), AmCPV-4 (*Antheraea mylitta*) and BmCPV-1 (*Bombyx mori*). (Arella et al., 1988; Qanungo et al., 2002; Rao et al., 2003). The RNA segments are composed of plus-strand mRNA and its complementary minus-strand in an end to end based-pair configuration with the exception of a protruding 5′ cap on the plus-strand

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(Furuichi and Miura, 1975). Based on the migration pattern of the genome segments on a 1% agarose or 3–5% polyacrylamide gel, CPVs are classified into 17 distinct electrophoretypes (Mertens et al., 1989, 1999, 2004b; Payne and Mertens, 1983; Payne and Rivers, 1976; Shapiro et al., 2005).

With the exception of a single isolate from mosquitoes, *Uranotaenia sapphirina* (UsCPV-17) (Shapiro et al., 2005), only lepidopteran cypoviruses have been characterized (Mertens et al., 2004b). However, there are many additional cypoviruses (>230) from other lepidopteran, dipteran, and hymenopteran hosts that have been reported but not classified (Andreadis, 1981; Mertens et al., 2004b). One of these is an unclassified CPV from the mosquito *Culex restuans* (Andreadis, 1986). Here, we report on the re-isolation of this CPV from *Cx. restuans* (named CrCPV) and present molecular evidence that CrCPV is most closely related to the recently isolated UsCPV-17 and propose that it be designated as CrCPV-17.

2. Materials and methods

2.1. Field collections and gross pathology of mosquitoes

Mosquito larvae were collected from various permanent water habitats in the environs of Alachua County, Florida, USA. *Cx. restuans* (the target host of this study) is a seasonal species in this region and is most prevalent during the winter months of January through March. Larvae were collected from the field sites following previously described protocols (Becnel et al., 2001) and returned to the laboratory for processing. Larvae were separated by species, and placed on a clear petri dish with a minimal amount of water for examination. Infected midgut cells were detected using a dissecting scope by viewing larvae from the ventral side on a dark background. The total number of larvae, the proportion of each species present, and the percent infection were recorded from each collection.

2.2. Virus production

Culex quinquefasciatus was used as the host in all laboratory bioassays because *Cx. restuans* has not been colonized. Groups of 3000 early fourth instar *Cx. quinquefasciatus* larvae (5 days old) were held in 500 ml water without food at 24 °C overnight, then exposed to 100 homogenized CrCPV infected cadavers in 10 mM $MgCl_2$ with food (0.2% alfalfa/pig chow 2:1) and held at 27 °C for an additional 24 h. The following day, the larvae and exposure media were transferred to trays to a final volume of 3 L ($MgCl_2$ concentration of 1.7 mM). Larvae were held for an additional 3 days and examined under a dissecting microscope for patent infections.

2.3. Purification of CrCPV

Groups of between 100 and 500 infected larvae were homogenized in 5–10 ml of deionized water and strained through a 400 mesh nylon screen. The filtrate was layered on top of an HS-40 Ludox continuous gradient and centrifuged at 16,000*g* for 30 min. The resulting band containing purified virus formed at an estimated density of 1.157 ± 0.002 (mean $\pm$ SE), $n = 6$. The viral band was recovered, washed in 0.1 mM NaOH three times, and held in 0.1 mM NaOH at 8 °C.

2.4. Cation assays

Groups of 100 mosquito larvae (third star) were exposed to purified virus (~10 larval equivalents (LE) of CrCPV) in 100 ml deionized water in the presence of 0 or 10 mM $MgCl_2$, and/or 10 mM $CaCl_2$, and 0.04% alfalfa/pig chow (2:1) for food. Controls included exposure of larvae to only 10 mM $MgCl_2$, 10 mM $CaCl_2$ or virus alone. The bioassays were repeated for a total of three trials. The larvae were examined 3 days after exposure under a dissecting microscope against a dark background and checked for visible signs of infection.

2.5. Ultrastructural studies (electron microscopy)

Dissected midguts of fourth instar larvae infected with CrCPV were processed for electron microscopy as described in Shapiro et al. (2004). Briefly, dissected midguts were fixed in 2.5% glutaraldehyde for 2 h, postfixed in 2% osmium tetroxide for 1 h, dehydrated in an ethanol series, and embedded in epon–araldite. Thin sections were stained in uranyl acetate and lead citrate, examined, and photographed at 75 kV.

2.6. Electropherotype analysis of CrCPV

A QIAampViral Mini Kit from Qiagen was used to extract genomic dsRNAs of CrCPV from purified polyhedra (Hagiwara et al., 2002; Rao et al., 2003). After precipitating, washing, drying, and resuspending the RNA in RNase free water, ~100 ng was analyzed on a 1% agarose gel. Ethidium bromide was integrated into the gel at a final concentration of 0.5 mg/ml. Genomic RNA from BmCPV-1, TnCPV-15, and UsCPV-17 was added to the gel to compare the electrophoretic profile of different cypoviruses. The sequences of BmCPV-1, TnCPV-15, and Seg-10 of UsCPV-17 (Accession No. AY876384) genomes are accessible in GenBank.

To compare structural protein profiles of CrCPV with that of UsCPV, purified virions were denatured by boiling in protein sample buffer and analyzed by electrophoresis using a standard 12% SDS–polyacrylamide gel (PAGE) (Laemmli, 1970).

2.7. cDNA synthesis, amplification by PCR, cloning, and sequencing

The conditions for cDNA synthesis, amplification by PCR, cloning, and sequencing are similar to the protocols published by Shapiro et al. (2005). Briefly, RNA was isolated using a QIAampViral Mini Kit (Qiagen) after purification of the CrCPV. The dsRNAs were then ligated to the anchor primer using T4 RNA ligase (New England Biolab). AMV reverse transcriptase (Promega) was used to synthesize cDNA. PCR was completed (Advantage 2 PCR Kit (Clontech)) using the primer 5-15-1 that was complementary to the anchor. The conditions for 30 cycles were 95 °C for 30 s and 68 °C for annealing/extension for 3 min.

PCR products were separated on a 1% agarose gel. The expected size of Seg-10 was ~900 bp. Seg-10 was then excised, purified, A-tailed by *Taq* enzyme (10 min at 72 °C with 200 nM dATP in 1× *Taq* buffer), and cloned into pGEM-T Easy vector. The clone insert from Seg-10 was amplified by PCR using SP6 and T7 primer pairs and was confirmed by the lengths of the PCR product and by restriction mapping. Four clones from Seg-10 were completely sequenced using SP6 and T7 primers with a dye terminator cycle sequencing ready reaction kit on a Beckman CEQ8000 system. In addition, the obtained sequences were used to design primers for direct sequencing of the RT-PCR products from Seg-10 (without cloning), thereby confirming the cloned sequence data.

2.8. Homology analysis

Homology searches of the nucleotide and predicted amino acid sequence of CrCPV were performed using BLAST (NCBI). Alignment of CrCPV Seg-10 to various CPV polyhedrins from GenBank was performed using Blast 2 sequences (bl2seq) (Tatusova and Madden, 1999) on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

3. Results

3.1. Field collection and gross pathology

Culex restuans infected with a cypovirus were first collected in Gainesville, FL, USA, in February, 1998. The infected midguts were visible through the cuticle on the larva's ventral side. White, irregular shaped regions restricted to the cytoplasm were present in the posterior midgut and in the gastric caeca. Infected larvae (Table 1) were found in six collections with an average infection rate of 21.1 ± 6.7% (mean ± SE). The number of larvae collected ranged from 4 to 140 larvae (38.2 ± 21.2). Cypovirus infections were also found in *Culex salinarius* in three of the collections (7.2 ± 6.4%), and *Culex nigripalpus* in one collection (1 of 5 larvae for 20% infection rate). *Culex territans* were present in three of the collections, but none were infected.

Table 1
Field collection of mosquitoes with CrCPV-17

Host	Number of collections	Larvae collected	Percent infections
Cx. restuans	6	38.2 ± 21.2	21.1 ± 6.7
Cx. salinarius	3	29.0 ± 15.4	7.2 ± 6.4
Cx. territans	3	15.7 ± 7.4	0
Cx. nigripalpus	1	5	20

3.2. Cation assays

Laboratory transmitted infections in *Cx. quinquefasciatus* were similar in appearance to naturally infected *Cx. restuans*. Patent infections were not produced in *Cx. quinquefasciatus* larvae when exposed to CrCPV in deionized water alone. However, significant infection levels (~30%) were obtained with the addition of magnesium during virus exposure (Table 2). The addition of calcium ions to the water in the presence of magnesium dramatically inhibited infection levels to ~2.0%.

3.3. Ultrastructural studies

Electron microscopic observation of infected larval midgut tissues showed viral infection in the cytoplasm of epithelial cells in the gastric caecae and posterior stomach. Non-occluded and occluded viral particles were not scattered throughout but were often localized in some areas of the cytoplasm (several per cell) (Figs. 1A and B). These areas generally lacked the normal cytoplasmic organelles and ribosomes, although other regions of the cells appeared unchanged containing all of the typical cellular organelles (Fig. 1A). Regions of virogenic stroma also lacked the typical cytoplasmic organelles and had an electron density close to that of ribosomes (Fig. 1B). Virus particles in the virogenic stroma were randomly distributed. Non-occluded virus particles (approximately 50 nm in diameter) consisted of a poorly defined capsid (icosahedral in shape, 32 nm in diameter) with projections (10–15 nm long) and an electron-dense core (darker than ribosomes and virogenic stroma, approximately 22 nm in diameter) (Fig. 1C). Virions were mostly occluded singly by the deposition of a crystalline polyhedrin matrix around individual particles, although mature inclusion bodies were pleomorphic with multiple virions (up to 20 virions in a cross section) and were formed by the coalescence of smaller inclusions (Figs. 1D and E).

3.4. Electrophoretic separation of dsRNA from CrCPV

The genome of CrCPV separated into 10 distinct segments on a 1% agarose gel (Fig. 2, lane 2). Comparison of

Table 2
Infection rate of CrCPV-17 in *Cx. quinquefasciatus*

$CaCl_2$ conc (mM)	CrCPV (LE)	Infection rate (%)	
		0 mM $MgCl_2$	10 mM $MgCl_2$
0	0	0	0
0	10	0	29.9 ± 21.4
10	10	0	0
10	10	0	1.7 ± 1.7

3rd instar larvae exposed and examined 3 days postinoculation.

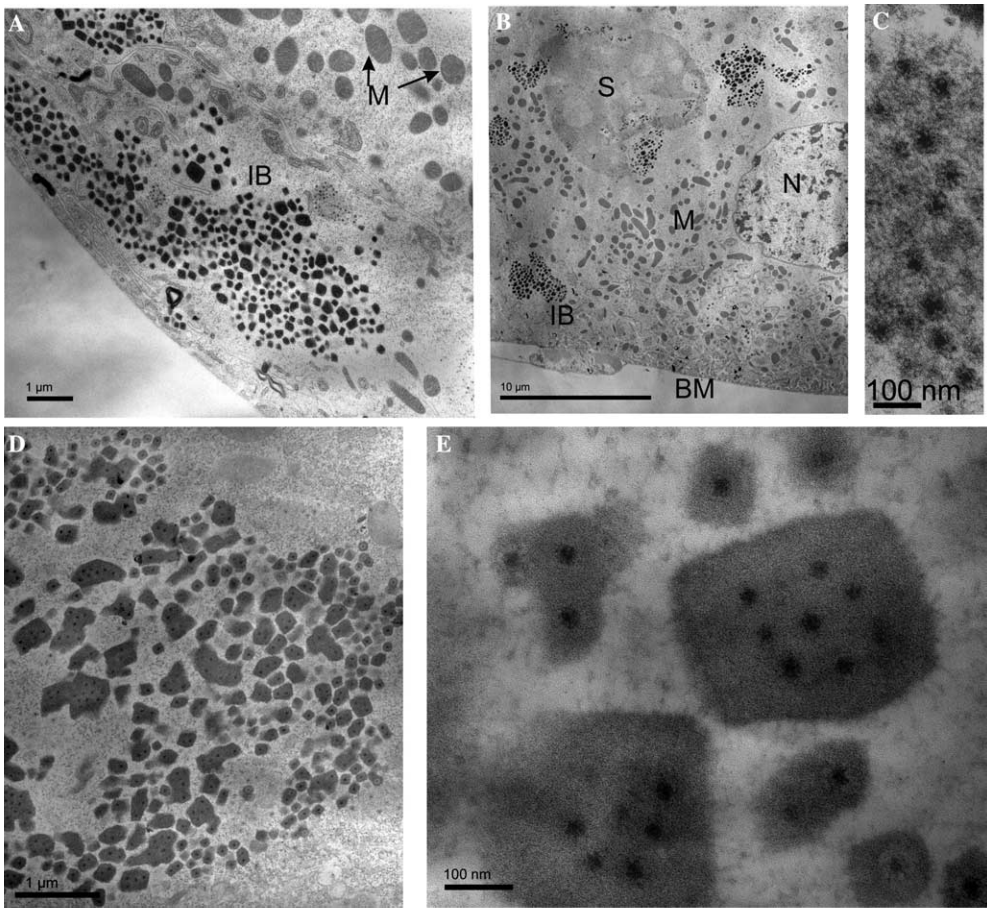

Fig. 1. Transmission electron micrographs of CrCPV-17 infections in the midgut epithelial cells of the posterior stomach of a field collected *Cx. restuans* larva. (A) An epithelial cell showing an area of accumulation of CrCPV-17 inclusion bodies (IB) where no typical cytoplasmic organelles are present. (B) An epithelial cell showing several areas of CrCPV-17 inclusion bodies (IB) and an area of virogenic stroma (S). (C) Non-occluded viral particles of CrCPV-17. (D) An epithelial cell showing an accumulation of large irregularly shaped inclusion bodies. (E) Inclusion bodies of CrCPV-17 containing multiple as well as single virions. M = mitochondria, N = nucleus, BM = basement membrane, IB = inclusion bodes, and S = Stroma.

CrCPV to the previously isolated mosquito UsCPV (Shapiro et al., 2005) (Fig. 2, lanes 2 and 3) clearly shows a similar electropherotype profile. The only noticeable differences between these two CPVs are the very small migration differences in segments 2, 3, 6, and 7 (asterisks in Fig. 2). In addition, comparison of the CrCPV genome segments to BmCPV-1 (Fig. 2, lane 1) and TnCPV-15 (Fig. 2, lane 4) illustrates that the RNA electropherotype from Diptera (mosquitoes) are quite different from those of Lepidoptera. The estimated basepairs (bp) of the CrCPV genome segments from Seg-1 to Seg-10 were 3870, 3750, 3580, 3300, 2400, 1900, 1850, 1500, 1500, and 900, respectively, as determined by comparison with markers and published sequence sizes for the genome segments of BmCPV-1 and TnCPV-15.

3.5. Sequence determination and analysis of segment 10

The sequence of CrCPV Seg-10 (Fig. 3) was found to be 892 nucleotides long (GenBank Accession No. DQ212785) and contained one open reading frame (ORF) from nucleotides 62–773. The ORF encodes a predicted protein of 237 amino acids with an estimated molecular mass of ~27 kDa.

When purified CrCPV was analyzed by SDS–PAGE, a major band with an apparent molecular mass very close to that predicted for the translation product (~27 kDa) from Seg-10 (Fig. 4, lane 2) was observed. The protein profile of CrCPV on the SDS–PAGE was similar to that for UsCPV-17 (Fig. 4, lane 3). Additionally, the theoretical isoelectric points (p*I*) for both CrCPV (6.12) and UsCPV-17 (6.08) were similar. The protein's secondary structure was predicted by GOR4 (Garnier et al., 1996) to entail a composition of 20.7% α helix, 26.6% extended strand, and 52.7% random coil. This was comparable to UsCPV-17, which had a predicted secondary structure of 21.9% α helix, 27.9% extended strand, and 50.2% random coil.

3.6. Comparison of RNA termini

In the family of *Reoviridae*, the terminal regions of genome segments are used to classify different virus species (Mertens et al., 2004a). This criterion was used in the present study to assess the similarity between CrCPV and UsCPV-17. The terminal sequence of CrCPV Seg-10 was 5′-AGAACAAA…CUACACU-3′, which was similar to

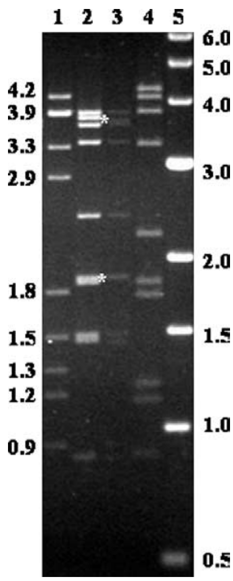

Fig. 2. Electrophoretic separation of various CPVs on a 1% agarose gel. BmCPV-1 (lane 1), CrCPV-17 (lane 2), UsCPV-17 (lane 3), TnCPV-15 (lane 4), and 1 kb marker (lane 5). The asterisks between lanes 2 and 3 indicate the visual differences between CrCPV-17 and UsCPV-17. The published segment sizes for BmCPV-1 (lane 1) are labeled to the left of the figure. The published sizes for TnCPV-15 (lane 4) are: Seg-1 = 4361 bp and Seg-10 = 897 bp.

the newly identified UsCPV-17 terminal sequence of 5′-AGAACUU….GUUACACU-3′ (Table 3). The five terminal bases at the 5′ end and six terminal bases at the 3′ end were identical. However, these terminal sequences shared no similarity with the terminal sequences of the seven other published *Cypovirus* species (types 1, 2, 4, 5, 14, 15, and 16). (Mertens et al., 2004b). For example, Seg-10 from CPV-5 family had a conserved termini sequence of 5′-AGUU…….UUGC-3′ while the CPV-1 family displayed 5′-AGUAA……GUUAGCC-3′.

3.7. Homology with other CPV polyhedrins

Alignment of the Seg-10 from CrCPV and UsCPV-17 nucleotide sequences showed an 83% identity (data not shown). Furthermore, comparing the amino acid sequences using BLAST (bl2seq) (Tatiana et al., 1999) of the predicted ORFs for CrCPV and UsCPV-17 Seg-10 revealed an 87% identity (Fig. 5). Alignment of the deduced amino acid sequence of CrCPV Seg-10 to those of other published cypoviruses showed low similarities to only CfCPV-16 (29%) and AmCPV-4 (23%) (Table 4). The remaining CPVs were not significantly similar to CrCPV.

4. Discussion

There are over 200 cypoviruses reported from lepidopteran, dipteran, and hymenopteran hosts that have remained unclassified due to the lack of genomic data (Andreadis, 1981, 1986; Federici, 1985; Mertens et al., 2004b). A characteristic electrophoretic migration pattern of the 10 dsRNA segments and their sizes are the major features used to characterize CPVs (Mertens et al., 2004b; Payne and Rivers, 1976; Rao et al., 2003). Currently, only 17 CPV species have been classified. Sixteen species are from Lepidoptera (Mertens et al., 2004b) and one is the newly discovered UsCPV-17 isolated from a dipteran host (Shapiro et al., 2005). CrCPV, first discovered by Andreadis (1986), is an example of a cypovirus that has remained unclassified due to the lack of molecular and genomic data. Comparison of the electropherotypes of CrCPV with UsCPV-17 (Fig. 2) revealed a striking similarity between the two genomes. Further comparison of UsCPV-17 and CrCPV to BmCPV-1 and TnCPV-15 electropherotypes illustrates that these mosquito CPVs are distinct from the lepidopteran CPVs both in migration patterns and size of the segments. These species parameters proposed by the International Committee of Taxonomy of Viruses (ICTV) (Mertens et al., 2004b) support placement of CrCPV into *Cypovirus*-17 group (strain CrCPV-17).

Molecular biology techniques, such as comparison of RNA sequences, serological comparisons of CPV proteins, cross-hybridization analyses of the dsRNA, or conserved terminal sequences among isolates in the same species, are alternative tools that may be used to support classifications of CPVs (Mertens et al., 2004b). Sequence analysis of the CPV genome Seg-10s, which are the viral polyhedron protein, shows high homology if isolates of CPVs are from a similar species group or low homology if isolates are from a different species group (Mertens et al., 2004b). For example, isolates of *Cypovirus*-1 and *Cypovirus*-5 exhibit nucleotide and amino acid sequence identities between 80 and 100% within each type (Table 4). Nucleotide and amino acid comparisons of CrCPV-17 with UsCPV-17 revealed an identity of 83 and 87%, respectively, (Fig. 5 and Table 4). However, the sequences in the table also illustrate that Seg-10 of CrCPV is substantially different when compared to the remaining CPVs. In addition, the conserved terminal regions of CrCPV are very similar to the terminal regions of UsCPV-17 (Table 3). Therefore, the sequence data also support the conclusion that CrCPV is an isolate of the *Cypovirus*-17 species group.

The inclusion bodies of CrCPV-17 are located in the cytoplasm of epithelial cells of the gastric caeca and posterior stomach. The developmental and ultrastructural morphology of CrCPV-17 is very similar to the description of an isolate from the same host but a different geographical location (Andreadis, 1986). Overall, the morphology of CrCPV is comparable to other cypoviruses isolated from mosquitoes. Inclusion bodies are formed when polyhedron is deposited around individual virus particles as previously demonstrated in an unclassified CPV from *Anopheles quadrimaculatus* (Anthony et al., 1973) and UsCPV-17 (Shapiro et al., 2005). In these species, the majority of inclusion bodies does not coalesce but remain as individual cuboidal particles that contain only one virion. For UsCPV-17, the size and arrangement of these inclusion bodies into paracrystalline arrays result in the midgut

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  1 agaacaaatgctaacattgaaattgaggtaagagaaactatctaagagaaacttaaccaaa

 62 atggcagatctatcactagctcgtcaacgcctggccaatgagtcagtcaacgaagcaccc
    M  A  D  L  S  L  A  R  Q  R  L  A  N  E  S  V  N  E  A  P

122 cgcgcatatgatgcgaacatggaacttgtcgttgtcgccgaatacccggagggacaatgt
    R  A  Y  D  A  N  M  E  L  V  V  V  A  E  Y  P  E  G  Q  C

182 aaatcattccacttcgccaatcctttcgtcgtcaaaggcgtcatcaaatcttcggaattg
    K  S  F  H  F  A  N  P  F  V  V  K  G  V  I  K  S  S  E  L

242 atgtgggacatcgacgacggacgtcaactgtctgaatatgacttgcagcgccgcattaac
    M  W  D  I  D  D  G  R  Q  L  S  E  Y  D  L  Q  R  R  I  N

302 ggctacgcagcgagtcattcaaacatgaaacaacgttcgtcggtcaaccgtatcccgcgt
    G  Y  A  A  S  H  S  N  M  K  Q  R  S  S  V  N  R  I  P  R

362 aagctatccttctatatgaggggtaacattgactggaacaaggtgtctattgacatccgc
    K  L  S  F  Y  M  R  G  N  I  D  W  N  K  V  S  I  D  I  R

422 ggaccaactggactatctaggcgccaaacggaagagtactctctggatcgtatccgtccc
    G  P  T  G  L  S  R  R  Q  T  E  E  Y  S  L  D  R  I  R  P

482 ccatgctcattcaagcgaaacaaactcatcgacttgccttcttgcggaggcaggtgtgaa
    P  C  S  F  K  R  N  K  L  I  D  L  P  S  C  G  G  R  C  E

542 aaggcttggttcgttgagctggacggctgcccggtgtcgatctcggtcctactgccccgg
    K  A  W  F  V  E  L  D  G  C  P  V  S  I  S  V  L  L  P  R

602 aacatgcataacggaattaacctttatgctggcccgctgctcggtaatgtgatcgaaggt
    N  M  H  N  G  I  N  L  Y  A  G  P  L  L  G  N  V  I  E  G

662 cttgacgtcgttccagagtgcactcaatggtttgacaactctcctgaattgtatgcttac
    L  D  V  V  P  E  C  T  Q  W  F  D  N  S  P  E  L  Y  A  Y

722 ttggctgagaactacggcatgaccatgcttgatcagttttctgttgtccactaggtgata
    L  A  E  N  Y  G  M  T  M  L  D  Q  F  S  V  V  H  -

782 catctcttagaagcaaaactccgagaaatctccggcctaagcaactgtgaagagatcaat

842 tttgaaagtacaatcccaatgtattaaacgtcagcatttaccaactacact 892
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Fig. 3. Nucleotide and predicted protein sequence of Seg-10 of CrCPV-17 (237 amino acids). The initiation and termination codons for CrCPV-17 are underlined. The translated amino acid is listed below the nucleotide sequence.

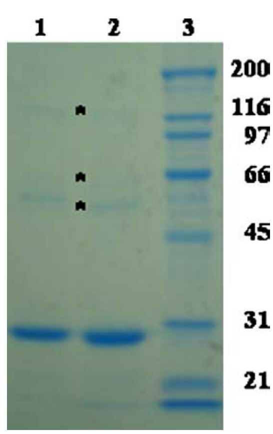

Fig. 4. SDS–PAGE analysis of purified CrCPV-17 and UsCPV-17. CrCPV-17 (lane 1), and UsCPV-17 (lane 2), Bio-Rad SDS Broad Range Standards in kilodalton (lane 3) were run on a 12% SDS–PAGE gel and stained with Gelcode Blue Stain Reagent (Pierce). Additional bands (asterisks) are located on the gel but are not as intense as the major band at ∼27 kDa.

Table 3
Comparison of Cypovirus segment-10 terminal regions

Virus strain	Terminal region sequences
CrCPV-17	5′-**AGAAC**AAA… …… ……………. C**UACACU**-3′
UsCPV-17	5′-**AGAAC**UU…………..……….. GU**UACACU**-3′
CPV-1	5′-AGUAA…..……….................. GUUAGCC-3′
CPV-5	5′-AGUU………………………………..UUGC-3′

Bold and underline sequences indicate conserved nucleotides.

expressing a unique blue iridescent color (Shapiro et al., 2005). In CrCPV-17 however, the majority of individual virus particles coalesce to produce large pleomorphic inclusion bodies (up to 1 μm) containing multiple virions which gives the midgut a white opaque color. A similar developmental pattern and appearance has also been reported for two unclassified CPVs in *Aedes cantator* (Andreadis, 1981) and *Aedes taeniorhynchus* (Federici, 1973a,b).

Previous studies by Andreadis (1986) showed that CrCPV was host specific to *Cx. restuans* and could not be transmitted to *Culex pipiens* or *Cx. territans*. However, the infection level of CrCPV in *Cx. restuans* was relatively low with an average of 10.5% (range = 4.8–15.6%) for vertical transmission and ∼17.5% for horizontal transmission. We document here that *Cx. quinquefasciatus* also supports development of this cypovirus. In addition, recent studies of

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UsCPV:    1 MADLSLARQRLTDESVNEAPRAYDANMELVIVAEYPEGQCKSFHFANPFVIKGVIKSSEL  60
            MADLSLARQRL +ESVNEAPRAYDANMELV+VAEYPEGQCKSFHFANPFV+KGVIKSSEL
CrCPV:    1 MADLSLARQRLANESVNEAPRAYDANMELVVVAEYPEGQCKSFHFANPFVVKGVIKSSEL  60

UsCPV:    1 MWDIDNGHQMSEYELQRSINGYAASHSNMRQRSAINRIPKKLSFYLRGNVDWNKASIDIR 120
            MWDID+G Q+SEY+LQR INGYAASHSNM+QRS++NRIP+KLSFY+RGN+DWNK SIDIR
CrCPV:   61 MWDIDDGRQLSEYDLQRRINGYAASHSNMKQRSSVNRIPRKLSFYMRGNIDWNKVSIDIR 120

UsCPV:  121 GPTGLSMRQTEEYSLDRIRPPCSYKRNKFVDLPSCGGRCEKAWYVELDGRPVSIAVIVPR 180
            GPTGLS RQTEEYSLDRIRPPCS+KRNK +DLPSCGGRCEKAW+VELDG PVSI+V++PR
CrCPV:  121 GPTGLSRRQTEEYSLDRIRPPCSFKRNKLIDLPSCGGRCEKAWFVELDGCPVSISVLLPR 180

UsCPV:  181 NMHNGINLYAGPLLGNVIEGLDTVPECTQWFDNAPELYAYHASNYGMTMLDQFSVIH 237
            NMHNGINLYAGPLLGNVIEGLD VPECTQWFDN+PELYAY A NYGMTMLDQFSV+H
CrCPV:  181 NMHNGINLYAGPLLGNVIEGLDVVPECTQWFDNSPELYAYLAENYGMTMLDQFSVVH 237
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Fig. 5. Segment 10 amino acid alignment of UsCPV-17 and CrCPV-17. The alignment of the UsCPV-17 (top) and CrCPV-17 (bottom) was performed using Blast 2 Sequences (NCBI). The middle is the consensus sequence with blank spaces representing no similarity and positive (+) representing similar amino acids. The sequences are 87% identical (207/237) and 95% positive (227/237).

Table 4
Segment 10 amino acid identities from various Cypoviruses

CPV's	Cr-17	Us-17	Bm-1	Dp-1	Ld-1	Am-4	Ap-4	Es-5	Ha-5	Op-5	Ld-14	Tn-15	Cf-16
Cr-17	x	87	—	—	—	23	23	—	—	—	—	—	26
Us-17	87	x	—	—	—	22	22	—	—	—	—	—	29
Bm-1	—	—	x	97	97	—	—	—	—	—	—	—	—
Dp-1	—	—	97	x	99	—	—	—	—	—	—	—	—
Ld-1	—	—	97	99	x	—	—	—	—	—	—	—	—
Am-4	23	22	—	—	—	x	100	—	—	—	—	—	29
Ap-4	23	22	—	—	—	100	x	—	—	—	—	—	29
Es-5	—	—	—	—	—	—	—	x	98	98	—	26	—
Ha-5	—	—	—	—	—	—	—	98	x	100	—	27	—
Op-5	—	—	—	—	—	—	—	98	100	x	—	27	—
Ld-14	—	—	—	—	—	—	—	—	—	—	x	21	—
Tn-15	—	—	—	—	—	—	—	26	27	27	21	x	—
Cf-16	26	29	—	—	—	29	29	—	—	—	—	—	x

"x" represents the comparison of the same Cypovirus.

Abbreviations for CPV's: Cr-17, *Culex restuans*-17; Us-17, *Uranotaenia sapphirina*-17; Bm-1, *Bombyx mori*-1; Dp-1, *Dendrolimus punctatus*-1; Ld-1, *Lymantria dispar*-1; Am-4, *Antheraea mylitta*-4; Ap-4, *Antheraea proylei*-4; Es-5, *Euxoa scandens*-5; Ha-5, *Heliothis armigera*-5; Op-5, *Orgyia pseudotsugata*-5; Ld-14, *Lymantria dispar*-14; Tn-15, *Trichoplusia ni*15; Cf-16, *Choristoneura fumiferana*-16.

mosquito baculoviruses have demonstrated that transmission can be enhanced by the divalent cations magnesium, barium, cobalt, nickel, and strontium or inhibited by calcium, copper, iron, and zinc (Andreadis et al., 2003; Becnel et al., 2001). Therefore, we tested the possible influence of divalent cations on the transmission of CrCPV-17 to *Cx. quinquefasciatus* larvae. The results not only indicated that CrCPV-17 transmission was enhanced in the presence of magnesium but also confirmed that calcium inhibited transmission. These data may potentially establish a transmission pattern of CPVs in mosquitoes since similar results were also obtained in transmission studies with UsCPV-17 (Shapiro et al., 2005).

Mosquito CPVs are horizontally transmitted in the larval stages with infections carried through to the adult stage where vertical transmission results in infected progeny (Andreadis, 1986; Shapiro et al., 2005). These routes of transmission for mosquito CPVs provide the potential to deliver and express genes of interest in adult mosquitoes. Since mortality is low and infections benign in larvae and adults, expression vectors or systems based on mosquito CPVs could potentially be used to deliver and express desirable genes (inhibitors, reporter genes, etc.) for laboratory studies or to control/monitor large population of disease-vectoring adult mosquitoes. Sequencing of the entire genomes of CrCPV-17 and UsCPV-17 is currently underway as well as methods for growing and manipulating these viruses in cell culture in an effort to evaluate this novel approach as a research tool with potential mosquito control applications.

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